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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Wallace A. LONGTON of Carlisle, Pennsylvania;
Christine A. Martey of New Brunswick, New Jersey; and
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For:

LACTONES OF CARBOXYLIC ACID POLYSACCHARIDES AND METHODS FOR
FORMING CONJUGATES THEREOF

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Enclosed for filing as a new patent application for the above-identified
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- [X] 1. a copy of the specification containing 30 pages
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inventor(s) pursuant to 37 CFR 1.63;
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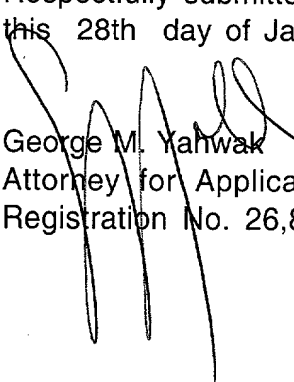
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- the appropriate filing fee of \$ and the
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- [] 9. _1_ sheet(s) of drawings;
- [] 10. Statement Under 37 CFR §1.821(f);
- [] 11. ___ page Sequence Listing;
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(ASCII) format; and
- [X] 13. This application claims priority from United States Provisional
Patent Application 60/117,673, filed on January 28th 1999.

Please accord this application a filing date of **JANUARY 28TH 2000**
in accordance with the provisions of 37 CFR 1.10.

Respectfully submitted on
this 28th day of January 2000,


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Ned D. Heindel et als.

Entitled:

LACTONES OF CARBOXYLIC ACID POLYSACCHARIDES AND METHODS FOR
FORMING CONJUGATES THEREOF

Filed:

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The documents deposited herein are:

- New Patent Application comprising
 - Letter of Transmittal
 - 1 page Abstract
 - 26 pages Description
 - 3 pages Claims;
 - 1 sheet Drawings;
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In accordance with the provisions of 37 CFR 1.10, please accord this international patent application the filing date of **January 28th 2000**.

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5 **LACTONES OF CARBOXYLIC ACID POLYSACCHARIDES AND METHODS FOR
FORMING CONJUGATES THEREOF**

10 **ABSTRACT OF THE DISCLOSURE:**

10 Disclosed are novel lactones of polysaccharide carboxylic acids and methods
for the preparation of a variety of conjugates therefrom, including metallo-
coordinated cisplatin (and carboplatin) as well as conjugates of ellipticinium,
aminoglutethemide, mitoxantrone, vitamin E, mitoguazone, cis 3-hexen-1-ol, and other
15 nucleophilic biologically effective agents.

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5 LACTONES OF CARBOXYLIC ACID POLYSACCHARIDES AND METHODS FOR FORMING CONJUGATES THEREOF

10 The formation of peritoneal or intraabdominal adhesions is a frequent and
often perilous derivative of general abdominal surgery, hernia repair, laparotomy,
peritoneal injury, or radiation therapy [see M. A. Weibel and G. Majno, "Peritoneal
adhesions and their relation to abdominal surgery," *Am. J. Surg.*,126:345-347 (1973);
S. J. Mathes and L. Alexander, "Radiation injury," *Surg. Oncol. Clin. North America*
5(4):809-824 (1996); and L. Holmdahl, B. Risberg, D. E. Beck, J. W. Burns, N. Chegini,
G. S. diZerega, H. Ellis, "Adhesions: pathogenesis and prevention-panel discussion and
summary," *Eur. J. Surg. Suppl.*,577: 56-62 (1997)]. Weibel's autopsy report of 752
15 patients who had undergone abdominal surgery revealed an adhesion rate of 67%.
Even surgical division of an adhesion to relieve a previously induced intestinal
obstruction may result in a recurrence of the adhesion in as many as 32% of the
cases [see N. L. Brightbill, A. S. McFee, and J. B. Aust, "Bowel obstruction and the long
tube stent," *Arch. Surg.*,112: 505 (1977)]. For 1992 the National Center for Health
20 Statistics reported 344,000 operations within the USA alone for repair of peritoneal
adhesions [see E. J. Graves, "1992 Summary," National Hospital Discharge Survey,
National Center for Health Statistics, U.S. Dept. of H.H.S., 249:7 (April 8, 1994)].
Fibrotic tissue damage resulting from radiation therapy is dose dependent and may
25 not be clinically evident for months or years after treatment [see Mathes, Supra].

These internal adhesions can become pathologic as a result of the anatomical
distortions which result. These distortions cause subsequent morbidities such as
intestinal obstructions, infertility, chronic pelvic pain, volvulus, or even hemorrhage.
Whether induced by radiation or surgery many techniques have been employed to
30 reduce the incidence of adhesion formation. These include adjusting the surgical
approach, or administration of antioxidants, hyperbaric oxygen, fibrinolytic drugs,

5 phospholipids, or barrier polymers [see H. Baeuml, U. Behrends, R. U. Peter, S. Mueller, C. Kammerbauer, S. W. Caughman, and K. Degitz, "Ionizing radiation induces, via generation of reactive oxygen intermediates, intercellular adhesion molecule-1 (ICAM-1)," *Free Radical Res.*, 27 (2):127-142 (1997); Holmdahl, Supra; and Mathes, Supra].

Polar, water-soluble, often anionic polymers (either as absorbable films
10 mechanically placed on site or as viscous aqueous solutions injected i.p.) seem to be the most successful. Some examples of these water-soluble anionic polymers - all of which have been shown capable of reducing adhesions - include sodium hyaluronic acid, sodium carboxymethyl cellulose, chondroitin sulfate, heparin, papain, and sodium polyacrylate [see A. Alponat, S. R. Lakshminarasappa, N. Yavuz, and P. M. Goh,
5 "Prevention of adhesions by Septrafilm™, an absorbable adhesion barrier," *Am. Surgery*, 63(9):818-819 (1997); J. W. Burns, K. Skinner, M. J. Colt, L. Burgess, R. Rose, and M. P. Diamond, "A hyaluronate based gel for prevention of postsurgical adhesions," *Fertil. Steril.*, 66(5):814-821 (1996); N. Cvetkovic, M. Nesic, V. Moracic, M. Rosic, "Design of a method for in vitro studies of polymer adhesion," *Pharmazie*,
20 52(7):536-537 (1997); E. S. Harris, R. F. Morgan, and G. T. Rodeheaver, "Analysis of the kinetics of peritoneal adhesion formation in the rat and evaluation of potential antiadhesive agents," *Surgery*, 117(6):663-669 (1995); G. Oelsner, R. A. Graebe, S. B. Pan, F. P. Haseltine, E. R. Barnea, H. Fakih, A. H. DeCherney, "Chondroitin sulphate: a new intraperitoneal treatment for postoperative adhesion prevention," *J. Reprod.*
25 *Med.*, 32(11):812-814 (1987); J. Ortega-Moreno, "Effects of TC7 associated to 32% dextran 70, heparin, and carboxymethylcellulose in adhesion prevention in the rat," *Arch. Gynecol. Obstet.*, 253(1):27-32 (1993); O. M. Parra, W. A. Saad, S. Ferri, L. Peduto, J. B. Ferraz-Neto, G. M. Dal Colletto, "Prevention of peritoneal adhesion formation with a combination of carboxylmethyl cellulose and papain," *Arq.*
30 *Gastroenterol.*, 28(2):63-68 (1991); and J. M. Seeger, L. D. Kaelin, E. M. Staples, Y.

- 5 Yaacobi, J. C. Bailey, S. Normann, J. W. Burns, and G. P. Goldberg, "Prevention of postoperative pericardial adhesions using tissue-protective solutions," *J. Surg. Res.*, 68(1):63-68 (1997)]. Ordinary dextran has been employed but appears to display a most minimal adhesion protective effect [see Harris, Supra].

10 Since treatment for malignancy is the most common medical reason for surgery or radiation therapy in the abdominal area, the combination of an antiadhesion pharmacology with an anti-tumor effect in a polymeric therapeutic adjuvant would represent a profound clinical advantage, and it is to this advantage that the present invention is directed.

15 Accordingly, the present invention discloses novel carboxy- and carboxymethyl-saccharide lactones (for example those derived from cellulose, starch, cyclodextrin, citosan, and pectin) and methods for the ring-opening of these lactones to prepare a variety of biologically-efficacious, i.e., having a biological response within the targeted end-user, conjugates thereof. These biologically efficacious conjugates are: therapeutics, including but not limited to metallo-coordinated cisplatin (and 20 carboplatin) conjugates and covalently-linked conjugates of ellipticinium, aminogluthemide, mitoxantrone, finasteride, vitamin E, alpha-difluoromethylornithine (DFMO), mitoguazone (also known as MGBG or methylglyoxalbisguanylhyazone) and other nucleophilic chemotherapeutics; imaging diagnostics such as saccharide bound chelating agents capable of binding radioactive metal ions for nuclear imaging 25 or paramagnetic metal ions for magnetic resonance imaging; fragrances for application in, for example, laundry or washing products; flavorings for application in, for example, foods and chewing gums; and property modifiers, i.e., thickeners, humectants, dispersants, in, for example, foods, paints, and other products. In short, the carboxy-functionalized and carboxymethyl-functionalized polysaccharide

5 compounds produced from the lactones according to the present invention have utility in a wide range of products.

As used herein, the term "therapeutic" includes treatment or prevention of any medical condition, i.e., for example, those conditions including, but not limited to, malignant and benign conditions, BPH, and endometriosis. The term "degree of
10 substitution" or "d.s." means the ratio of attached molecules per each repeating monomer unit, usually glucose or galactose, in each of the saccharide carriers employed in the present invention.

The present invention describes that a reactive lactone of carboxymethyl cellulose can be linked directly to anti-tumor drugs containing amino or hydroxy
15 functions, or to any nucleophilic species, without use of the typical conjugation activators or chemical promoters which may leave unproductive, N-acyl rearranged residues from the promoter itself on the polymer backbone. In this context the terms "conjugation activators" or "chemical promoters" includes carbodiimides, mixed anhydrides, homo and hetero-bifunctional couplers and related agents effecting small
20 molecule to macromolecule attachment as described in Bioconjugate Techniques [by Greg T. Hermanson], Academic Press (1996).

The method of lactone opening according to the present invention provides two inherent and marked advantages: Since in the art most couplings are traditionally performed in aqueous media, the inherent instability to water of the coupling
25 promoters must be compensated by use of an excess quantity [see M. A. Gilles, A. Q. Hudson, and C. L. Borders, *Anal. Biochem.*, 184:244-248 (1990)]. In addition, a chemical residue of the reagents intended to promote coupling often remains on the macromolecule backbone [S. S. Wong, *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press, (199) pp. 122-123] and is difficult to remove. Thus, even if both
30 the polysaccharide carrier and the agent being released from it are acceptable as

5 being safe as reflected either by inclusion on the GRAS list or on the FDA's list of approved pharmaceuticals, the altered carriers containing the residues of the non-productive coupling agents may have an adverse, or at least an unknown, pharmacology.

10 The chemical process according to the present invention for attaching the small molecules to the polysaccharide lactone never utilizes all the available lactones, and therefore upon hydrolysis, and pH adjustment, one can have pendant acid carboxyls or carboxylate salts. As noted above, these anionic polysaccharide carboxylates are useful in antiadhesion therapy and by the conjugation with pharmaceuticals according to the present invention, and thus possess the supplemental utility of cancer
15 chemotherapeutic healing of abdominal malignancies which have been surgically excised or treated by radiation *in situ*. Lactones of many other polysaccharides can be prepared and coupled according to the present invention to biologically-significant small molecules for therapeutic, flavoring or fragrance applications without any intervening chemical promoter.

20 Polysaccharides that have yielded internal lactones when tested according to the present invention include carboxymethyl cellulose, carboxymethyl cyclodextrin, carboxymethyl starch, carboxymethyl chitosan, pectin, and carboxy starch. The carboxymethylated saccharides are widely reported and synthesized by traditional condensation of the parent carbohydrate with chloroacetic acid in aqueous base. It is
25 critical that the degree of substitution of carboxymethyl per monomeric carbohydrate unit not exceed 1.2. Optimum lactonization requires that the carboxylic group be statistically able to grip a proximal hydroxyl.

While carbohydrate derivatives are well-known to those skilled in this art, it is best to note alternative names, and in some cases, commercial suppliers.

30 Carboxymethylcellulose sodium salt also known as SCMS or CMCS is available from

5 Hercules Inc. (Wilmington, DE); pectin potassium salt is available from Sigma (St. Louis, MO) (fruit pectin conventionally sold to the public for use in home canning is also satisfactory if purified as noted herein; O,N-Carboxymethyl, O-carboxymethyl, and N-carboxymethylchitosan are available from CarboMer (Westborough, MA), and N-carboxymethylchitosan is available from V-Labs Inc. (Covington, LA) (these chitosan
10 products can also be found in the literature as glucosamine polymer, carboxymethylated on O-, N-, or mixed O,N- specified); carboxymethyl starch is also known as starch glycolate or as starch carboxymethyl ether and is available as its sodium salt from Penwest Pharmaceuticals (Patterson, NY) or National Starch Inc. (Bridgewater, NJ); carboxymethyl alpha- and beta-cyclodextrins are available as the
15 free acids from CarboMer; carboxy-starch is a research-grade product produced by partial oxidation of the C-6 primary hydroxyl on starch by National Starch Inc.

After lactonization according to the method of the present invention described more fully herein, and subsequent ring-opening by a nucleophilic small molecule, each of the unique polymeric conjugates according to the present invention is capable of
20 releasing the 'active ingredient'. Furthermore, by controlling the effective degree of substitution (i.e., drug or other 'active ingredient' moieties per repeating glucose unit) at the time of synthesis, one can adjust the period of *in vivo* release of such conjugated compounds. The carboxy and carboxymethyl polysaccharide compounds made in accordance with the method of the present invention i.e., those compounds
25 to which have been attached an appropriate small 'active ingredient' molecule, have uses in areas outside of well outside of therapeutics, including in body implants, and in formulations for laundry products, chewing gum, food processing, and paint improvers.

In a more detailed description of the present invention, a process for the
30 preparation of saccharide lactones, chemotherapeutic and biologically-active

5 conjugates prepared from these lactones, and specific application of the salts prepared from drug-bearing lactones to chemoprophylaxes of adhesions arising in oncologic therapies according to the present invention is more fully described below.

10 In the biologically-active agents having therapeutic activity, two major classes of chemotherapeutics with established efficacy against malignancies of the abdominal regions, were selected for linkage to carboxymethyl cellulose as pro-drugs (as conventionally understood). These chemotherapeutics are (1) those metallo complexes whose attachment is coordinated (cisplatin, carboplatin), and (2) those capable of covalent attachment to the carboxyl function by a hydrolyzable bond (e.g. ellipticine, mitoxantrone, aminoglutethemide, vitamin E, mitoguazone). Chemical
15 coupling of the biologically-active agent (pharmaceutical) to the lactone provides a sustained release formulation (a pro-drug) which is released both with and without enzymatic action.

20 Sodium carboxymethyl cellulose, carboxymethyl cellulose (acid form), and the in situ hydrolyzed carboxymethyl cellulose (lactone form) was used to demonstrate linkage according to the present invention for the exemplified metallo-coordinated cisplatin.

Conjugates of fragrance and flavor components were used to demonstrate linkage of biologically-active compounds, in addition to the chemotherapeutic examples according to the present invention.

25 It is clear that saccharide lactone chemistry according to the present invention provides a more than acceptable method for the preparation of pro-perfumes and pro-flavors. In this application a pro-perfume is a chemical wherein the biologically-active ingredient is a molecule possessing useful fragrance properties 'tethered' or conjugated to a polysaccharide from which it demonstrates prolonged release either
30 with or without enzymatic action. A pro-flavor is the polysaccharide 'tethered' or

5 conjugated to a polysaccharide carrier of which it possesses useful flavoring properties when released, with or without enzymatic action, from the carrier.

Also described is the employment of cis-3-hexen-1-ol (or "leaf alcohol") 'tethered' or conjugated to a polysaccharide carrier as exemplary of the attachment of a further flavor or fragrance ingredient according to the present invention.

10 Accordingly, it is an aspect of the disclosure of the present invention to describe a series of lactones of polysaccharide carboxylic acids.

It is another aspect of the disclosure of the present invention to describe a method for preparing of a variety of conjugates from the series of lactones of polysaccharide carboxylic acids.

15 These and other aspects of the present invention can be deduced by those skilled in the art to which it pertains by reference to the following examples and figure, and description. The following examples are thus provided for purposes of clarity in order to more fully describe and demonstrate the methods by which the lactones and conjugates according to the present invention are prepared. However, these
20 examples are not meant to be limiting in any manner, and modifications and adaptations may be made to provide other routes or end products, all of which are to be considered to be within the scope of the present invention.

With regard to FIGURE 1, there is shown the controlled release of Cisplatin from CMC/CMD Cisplatin complexes made and tested according to the present invention.

25

EXAMPLE 1

Preparation of the Lactones

a) Purification of starting materials

It is preferred that all saccharide acids be purified, finely-powdered, anhydrous carboxylic acids with minimal sodium or potassium carboxylate content. Only the free
30 acid form of the carbohydrate generates a lactone under the conditions according to

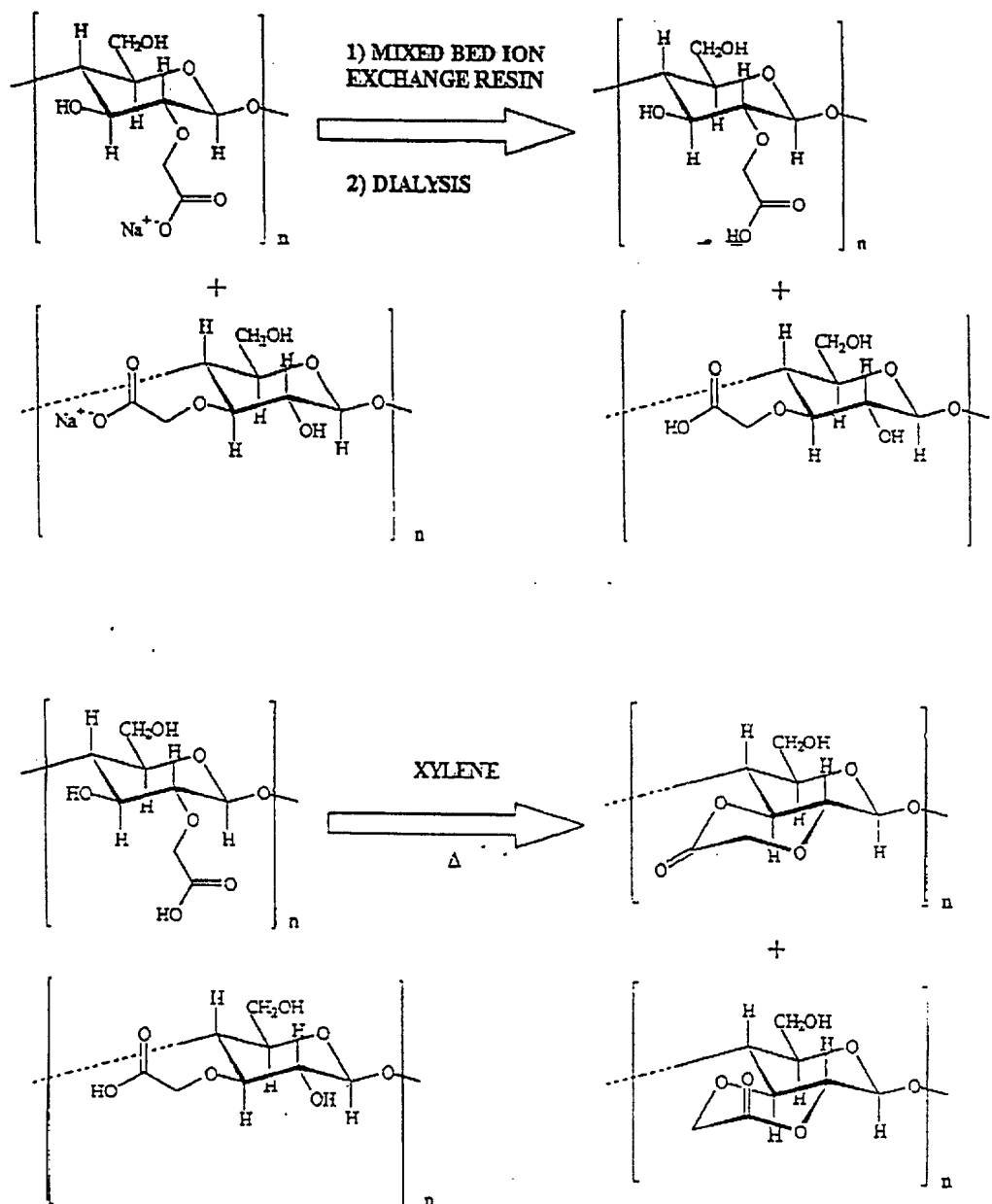
the present invention. To obtain effective lactonization, all starting materials (whether indicated as free acids or as salts by method of synthesis or by label description on commercial materials) were dissolved in distilled, deionized water and passed over a mixed bed resin ion exchange column. While other appropriate columns would be satisfactory, the Sigma Mixed Bed Resin TMD-8 column was selected for this purpose:

The eluant was charged to a dialysis bag (Sigma, 12,000 molecular weight exclusion), and dialyzed against distilled water for three to five days with replacement of the external water every 24 hours. The contents of the dialysis bag were *evaporated in vacuo* and lyophilized for 24 hours. FT-IR spectra showed no trace of carboxylate anion ($\text{C}=\text{O}$ ca 1610 cm^{-1}) but only the free carboxylic acid forms ($\text{C}=\text{O}$ in the range of $1720\text{-}1645\text{ cm}^{-1}$ depending on extent of internal hydrogen bonding) of the polysaccharides. Grinding, ball-milling, or "wiggle-bug" reduction to a fine powder was performed and the samples were held under vacuum over a drying agent until lactonization was performed.

b) Lactonization

Lactonization was carried out by thermal dehydration in an anhydrous non-nucleophilic solvent. The acid can be lactonized either as a suspension of the insoluble acid or as a solution or partial solution. Well-stirred media such as refluxing mixed xylenes (bp $138\text{-}144\text{ }^{\circ}\text{C}$), toluene (bp $109\text{-}110\text{ }^{\circ}\text{C}$), diglyme (bp $162\text{ }^{\circ}\text{C}$), and acetonitrile (bp $82\text{ }^{\circ}\text{C}$) perform satisfactorily. Degree of substitution (d.s.) of available $-\text{COOH}$ per repeating monomeric carbohydrate unit in the saccharide acid should not exceed 1.2 although much lower d.s. (0.25 to 0.80) perform well. Carboxymethyl moieties lactonize more extensively than the less flexible, more constrained, directly attached carboxylic acid moieties such as found in pectin acid and 6-carboxystarch.

- 5 A chemical structure 'flow chart' of these two general reactions for obtaining the lactonization according to the present invention is:



5 In the above structures, 'n' may normally be an integer from 500 to 2000, preferably between 1000 and 1500. However, the exact number of repeats is not critical to the present invention, and thus 'n' may vary over a wide range, depending upon the characteristics of the final product sought. The exact range is well within the skill of those in the art to determine given the description of the present invention
10 herein and the knowledge of what specific properties of the final conjugate are desired for a specific use.

Utilizing the experimental method described below, carboxymethyl cellulose was completely lactonized, carboxymethyl dextran was about 90% lactonized, and pectin acid was 50-70% lactonized.

15 As recognized by those skilled in the art, lactones can be recognized by altered physical properties compared to their starting acids. Carboxymethyl cellulose lactone, for example, deposits from the lactonization solvent as a film which can be pulverized to a yellowish-white solid that is virtually insoluble in water; carboxymethyl dextran lactone was collected as a water-insoluble white solid.

20 In their infrared spectra all lactones display a long wave length C=O between 1740 and 1760 cm^{-1} . On a high resolution FT infrared spectrophotometer, the band envelope of the lactone C=O stretch is often seen to consist of several closely spaced absorptions presumably reflecting that several different specific lactone structures are present. Lactones open and dissolve in aqueous base. The general lactonization
25 synthetic method requires heating about 1 gram of the finely pulverized acid in 50 ml of vigorously stirred anhydrous diglyme for 24 hours. Evaporation and filtration yield the lactone. While this general method is applicable to all carboxylic acid saccharides according to the present invention, we offer the following, more-specific examples.

EXAMPLE 2

Preparation of Carboxymethyl Cellulose Lactone (CMCL)

With covalent attachment of small molecules to carboxyl groups by the traditional carbodiimide-promoted coupling, one often experiences a substantial number of unproductive, N-acyl rearranged residues attached to the polymer backbone [Heindel *1994]. These residues have the potential to contribute to the immunogenicity and the toxicity of the saccharide polymeric carrier. A solution to avoid such chemical residues of the carbodiimide promoters is to not utilize them in the first place. In the case of carboxymethyl dextran a highly reactive internal lactone could be prepared [Heindel *1994] and employed in coupling without any promoting agents being present.

The hitherto unknown lactone of carboxymethyl cellulose (CMCL) provides a reliable coupling promoter for a wide variety of amino- and hydroxyl-containing biologically important molecules.

Carboxymethyl cellulose (free acid) was purified as previously described. The white flaky solid (1.0 g) was pulverized to dust in a wiggle-bug, suspended in 60 ml of anhydrous diglyme (or xylene), and heated at 150 °C for 24 hrs. The solvent was evaporated to 25 ml, chilled, and the lactone filtered off as a water-insoluble off-white solid. This solid was filtered and washed quickly twice with 10 ml each of cold water. The resulting product was then dried *in vacuo* by lyophilization.

If all the solvent used in the lactonization were evaporated to dryness in a rotary vacuum evaporator the lactone can be obtained as a film clinging to the walls of the vessel. A characteristic C=O stretch was seen at 1750 cm⁻¹ using FT-IR and is indicative of this lactone.

The route to the synthesis of this lactone is illustrated above wherein the sodium carboxymethyl cellulose is converted to the acid and the acid to the lactone.

- 5 The process of lactonization can be followed by the infrared C=O shift from acid (ca 1720 cm^{-1}) to lactone.

EXAMPLE 3

Preparation of Pectin Lactone

- 10 A suspension 2.0 g of purified, dried, finely-pulverized pectin acid was prepared in 70 ml of anhydrous toluene and heated with stirring at reflux for 24 hr following the general procedure described above. Evaporation of the solvent yielded a water-insoluble, gummy, semi-solid whose infrared spectrum revealed lactone (1748 cm^{-1}) and nonreacted acid (1680 cm^{-1}) in an intensity ratio of 70/30 lactone/acid. While the pectin acid could not be driven to a higher lactone content, this lactone could be
15 ring-opened with nucleophilic small molecules (i.e., primary and secondary amines and alcohols). Alternatively, *vide infra*, it was possible to optimize the loading of the small molecule onto pectin by an *in situ* generation and ring-opening of the lactone.

EXAMPLE 4

Preparation of Carboxymethyl Starch Lactone (CMSL)

- 20 Sodium carboxymethyl starch (1.0 g) was converted to the free acid, purified, dried, and pulverized as described above. It was lactonized by refluxing in 60 ml of anhydrous diglyme, isolated and purified as described above. FT IR spectra on the sodio salt displayed the carboxylate $-\text{COO}^-$ at 1620 cm^{-1} and on the purified acid displayed the C=O at 1717 cm^{-1} . Lactonization after 24 hr reflux was >90% complete
25 and the new lactone C=O was evident at 1742 cm^{-1} .

EXAMPLE 5

Preparation of carboxymethyl cellulose-cisplatin conjugate from the acid-form of the polymer

- 30 In complexometric binding of platinum (and indeed other metal-containing

5 complexes) to carboxylic acid ligands, ion concentrations may be critical. For example, it has been reported that cisplatin associates best with carboxylic residues if the sodium ions have been removed and all the carboxylic moieties are in the acid form [B. Schechter et al., *Cancer Biochem. Biophys.* 1986, 8: 277-287]. Therefore, additional time and effort is needed to convert the clinical grade of sodium
10 carboxymethyl cellulose (SCMC) to the non-sodio containing free acid. In accordance with the present Example 5, there is described the preparation of the cisplatin complex with $-\text{COONa}$, with COOH , or with in situ hydrolyzed lactone. The sodium carboxymethyl cellulose (SCMC) has a molecular weight of 250,000 and a degree of substitution (ds) of 0.8 to 0.9.

15 2.5 grams of sodium carboxymethyl cellulose (SCMC) was dissolved in 100 ml of distilled/deionized water by heating and agitation at ca 80-90 °C for approximately 10 minutes. The solution remained homogeneous on cooling to room temperature after which it was passed through an ion exchange resin column [Sigma: Mixed Bed Resin TMD-8], dialyzed [Sigma: membrane 12,000 mw exclusion] for three days with
20 three exchanges of water, evaporated in vacuo to about one-fifth the volume (ca. 20 ml). The resulting solution was then divided into two equal 10 ml portions one of which was lyophilized, dried to constant weight, and weighed. This technique was used to determine the number of grams (or moles) of the cellulosic polymer in the remaining aqueous aliquot.

25 In a separate sequence, 15-30 mg of cisplatin was dissolved in 1 to 2 ml of distilled/deionized water by briefly heating and agitating at ca 80-90 °C. A pale yellow homogeneous solution resulted. The solutions of cisplatin and carboxymethyl cellulose were then mixed in a volume ratio to insure that a mole ratio of 10/1 cisplatin/ OCH_2COOH was employed. Moles of carboxyl moieties on the cellulose were
30 determined from the known degree of substitution. The solution was sealed and

5 stirred at room temperature for 24 hours during which process a clear, nearly
colorless, solution resulted. Dialysis for removal of unbound cisplatin was carried out
against distilled/deionized water for five days with two water exchanges. After
dialysis was completed the solution within the bag was diluted with distilled/deionized
10 water to 25.0 ml. Since there is no loss of the carboxymethyl cellulose in the
purification process and since the final fluid volume is known, one can calculate the
moles of cisplatin to moles of the polymer by experimentally determining the amount
of bound cisplatin. Exhaustive dialysis in this fashion removed sodium ions and
produces the final polymer as carboxymethyl cellulose (CMS)-cis-platinum adduct.

15 An established analytical method for cisplatin bounded to polymeric systems
[Schechter-1986] was employed in which o-phenylenediamine was utilized as a
quantitative chromogen for a complex which was read at 703 nm against a
calibration standard curve. A typical conjugate prepared in this fashion has 40-50
millimoles of cisplatin per mole of CMC. The degree of substitution can be varied over
a wide range of 20 to 400 millimoles of cisplatin (or carboplatin) per mole of CMC by
20 control of the initial reacting ratio of the drug to carboxymethyl functions.
Furthermore, one is not limited to the use of a polymer whose (d.s.) is as low as 0.8
to 0.9 and carboxyl loads of 1.4×1.8 are serviceable. With higher (d.s.) of carboxyls
one achieves higher (d.s.) of cisplatin (or carboplatin) linkage.

EXAMPLE 6

25 Preparation of carboxymethyl cellulose-cisplatin conjugate from the
sodio salt-form of the polymer

2.5 grams of SCMC was dissolved in 100 ml of distilled/deionized water by
heating and agitation at ca 80-90 °C for approximately 10 minutes. The solution was
then cooled to room temperature and evaporated in vacuo to about 20 ml. It was
30 then mixed with a cisplatin solution as described above (with the understanding that

5 the ratio of platinum to carboxyl and of carboxyls per repeating glucose moiety can be varied widely) and treated in the same manner as the free acid. No significant differences in load of cisplatin/cellulose unit were observed whether commencing with the free acid or the carboxylate salt.

EXAMPLE 7

10 Preparation of carboxymethyl cellulose-cisplatin conjugates from the lactone of carboxymethyl cellulose (CMCL)

It is also possible to utilize the internal lactone (synthesized as described above) by heating, with agitation, at between about 80-90 °C a suspension of 2.5 g of the CMCL mixed with an appropriate amount of cisplatin as described above. The lactone, which is incompletely soluble in water, dissolves and reacts with the cisplatin apparently as its carboxylic residues are hydrolytically generated. No sodium ions and no base need be present in this drug-loading process. These are important features and aspects of utilizing a lactone in forming such conjugates. When commencing with the lactone, the same load of cisplatin/cellulose was achieved as when using the free acid or the carboxylate salt.

Although the procedures described above were carried out with various chemical forms of carboxymethyl cellulose, the techniques are applicable to other carboxy- and carboxymethylated polysaccharides and their lactones as described herein. For example, with carboxymethyl dextran, its sodio-salt, and its lactone [preparation described Heindel-1994], comparable results in (d.s.) per mole of glucose residue were obtained.

EXAMPLE 8

Bioavailability of bound drug

30 The cisplatin bound polymer according to the method of the present invention to the cellulose carrier as described above was established as being bio-available both

5 by enzymatic action (freshly drawn rat serum) and by spontaneous hydrolysis (pH 7.4 phosphate buffer). In this procedure, 1.0 ml of rat serum was mixed with 1 ml of cisplatin-CMC complex (of known concentration and known d.s.) and incubated at 37 °C for 80 hours. The cisplatin continued to be released from the polymer for the entire time span (and beyond) for both the carboxymethyl cellulose conjugates (CMC)
10 and the carboxymethyl dextran conjugates (CMD).

At the times indicated in Figure 1, samples were spun against a molecular weight cut-off barrier of 10,000 in a Centricon ultracentrifugation tube with a platinum analysis performed on the filtrate according to accepted testing protocols. The half-life for drug release in contact with serum was determined to be approximately
15 8.3 days, and the half-life for drug release in phosphate buffer was determined to be approximately 5.8 days. The drug was released from the dextran carrier in serum with a half-life greater than 16 days.

With specific regard to the bioavailability of drugs from the conjugates according to the present invention, in the experimental fashion described above for
20 measuring the controlled release of cisplatin from the polymer, each of the chemotherapeutics polymer conjugates was evaluated. In freshly-prepared mouse serum the enzyme-mediated release rates of ellipticinium, aminoglutethemide and mitoxantrone were quantified at their ultraviolet maxima according to recognized protocols, and fell in the range of 4-8 day half lives.

25

EXAMPLE 9

Conjugation of nucleophilic biologically-active substances to the lactones.

Any amino or hydroxy containing biologically-active compound can be conveniently linked to the lactone of carboxymethyl cellulose (CMCL), the lactone of carboxymethyl dextran (CMDL), the lactone of carboxymethyl starch (CMSL), the
30 lactone or pectin, or any of the other lactones according to the present invention.

5 Such biologically-active substances may be pharmaceuticals including but not limited to adriamycin, daunomycin, gemcitabine, bleomycin, 6-mercaptopurine, 5-FU, mephalan, ellipticine (and ellipticiniums such as ellipticinium bromide), mitoguazone, aminoglutethimide, squalamine, mitoxanthone, alpha-difluoromethylornithine, podophyllotoxin, and methotrexate.

10 In addition, such biologically-active substances may be flavoring alcohols including but not limited to leaf alcohol (cis-3-hexen-1-ol), menthol, acetoin (3-hydroxy-2-butanone), thymol, vanillin, and methyl salicylate. Attached as a pro-flavor to gum, candy, food, or any nutritional or non-nutritional product placed in the oral cavity in the form of a conjugate of the saccharides according to the present
15 invention, these chemicals sustain their biological activity (i.e., providing flavor) over a prolonged time whether being released from the saccharide with or without enzymatic action.

Furthermore, the biologically-active substances may also be fragrance compounds including but not limited to leaf alcohol (cis-3-hexen-1-ol), phenylethyl
20 alcohol, 3-methyl-5-phenyl-1-pentanol, 2-methyl-5-phenyl-1-hexanol, 1-hexanol, 1-decanol, 1-dodecanol, 3,7-dimethyl-1-octanol, isononanol (i.e., 3,5,5-trimethylhexanol and isomers), 2,2-dimethyl-3-phenyl-1-propanol, nopol, anisic alcohol, benzyl alcohol, 2-cyclohexylethyl alcohol, 2,4-dimethylcyclohexylmethanol, beta-methylphenylethyl alcohol, hydroxycitronellol, isocyclogeraniol, 3-hydroxymethyl-2-nonanone, 4-
25 isopropylbenzyl alcohol, 3-phenylpropanol, and others.

The only limitation on structure must be that the nucleophilicity and steric accessibility of the -NH or -OH functions in these biologically-significant molecules be sufficient to ring-open the saccharide lactones.

5 The following examples describe the versatility of the conjugates according to the present invention, and provide procedures for linking five pharmaceuticals (pro-drugs) and one perfume flavorant (pro-fragrance and pro-flavor).

EXAMPLE 10

Conjugation of electrophilic biologically-active substances to the lactones.

10 Direct condensation of any small molecule with the lactones according to the present invention requires that the incoming reactant be a nucleophile. Amines and alcohols readily open the lactones. For those cases in which it is desirable to attach an electrophile, a bridging bi-functional nucleophile must be inserted between the polysaccharide lactone and the condensing electrophile one wishes to attach;
15 hydrazine is one such linking agent that fulfills this bridging function.

20 All lactones react rapidly and in high efficiency with hydrazine (all lactones present are opened). Illustrative of this process was the condensation process in which 10 mg of CMCL, 5.0 ml of hydrazine hydrate, and 3.0 ml of water were mixed. The lactone quickly dissolved. The solution was allowed to stand for 3 hrs, diluted with 20 ml of water and dialyzed for 5 days with 3 changes of water. The solid hydrazide obtained on evaporation and lyophilization displayed FT IR bands at 1058 and at 1594 cm^{-1} characteristic of $-\text{CO}-\text{NH}-\text{NH}_2$. Combustion analysis for nitrogen gave 6.09% N which translates to a d.s. of 0.48. Subsequently, vitamin E (as alpha-tocopherol sulfo-N-hydroxysuccinimido succinate) and N,N,N',N'',N'''-diethylenetriaminepentaacetic acid (as DTPA bis-anhydride) were coupled to this
25 hydrazide (*vide infra*).

Condensing the nucleophiles according to the present invention is described below:

EXAMPLE 11:

Preparation of CMC-Aminoglutethimide conjugate.

CMC-lactone (CMCL) according to the present invention was prepared as previously described, and was opened by the amino function on the aminoglutethimide moiety. 360 mg of aminoglutethimide were dissolved in 40 ml of anhydrous acetonitrile and 336 mg of CMCL were added to form a suspension. The suspension was refluxed for 18 hrs under argon after which the reaction was cooled and the excess solvent removed under reduced pressure. Excess drug was removed by dissolving the cream colored flakes in 3 ml 0.1N NaOH, adding 7 ml of distilled water and dialyzing the solution against distilled water for 5 days with 2 changes of water. The small quantity of aqueous base opens the residual lactone moieties which had not undergone reaction with the drug. After dialysis, the retentive (sample in bag) was concentrated under reduced pressure and final drying done by lyophilization. The proton NMR and FT-IR show clear evidence that both drug and polymer are present. As was noted with cisplatin, the ratio of available drug per available lactone permits variation in the degree of substitution which is determined, in this case, by combustion analysis for % nitrogen.

¹H NMR (D₂O) 0.72 (t), 1.76 (m), 2.15 (m), 2.25 (m), 2.9-4.3 (Br), 6.85 (DD), 6.91 (DD). FT-IR (KBr pellet): 1730 cm⁻¹ (acid), 1650 cm⁻¹ (Br, amide); mp > 300 °C; d.s. = 0.10 by method above; if reactant ratios are varied d.s. values of 0.01 to 0.5 are obtained when the original saccharide had 0.85 carboxymethyls/glucose

EXAMPLE 12

Preparation of CMC-ellipticinium conjugate

120 mg of 2-(4-hydrazino-4-oxobutyl) ellipticinium bromide (yellow powder) was dissolved in 25 ml anhydrous acetonitrile, and 235 mg CMCL added leading to formation of a suspension. The reaction was stirred and refluxed under argon for 15

5 hr and the excess solvent removed by evaporation in vacuum. The deep yellow flakes were only partially soluble in water due to nonreacted lactone. A few drops of 0.1N NaOH achieved complete solubility by opening the remaining lactone as was done previously. The excess ellipticine was dialyzed against water for 5 days with 2 changes of water. The yellow retentive was concentrated under reduced pressure and dried via lyophilization. Quantification for (d.s.) was performed against an ultra-violet calibration curve prepared with the ellipticinium standard at the indicated maximum. Proton NMR and FT-IR showed the presence of drug and polymer. The 0.13 load of ellipticinium on the conjugate made full interpretation of its peaks impossible with the larger contribution of the polysaccharide. Aromatic resonances in the conjugate from 6.6 to 8.9 matched the aromatic protons in the original drug. C-H resonances from the carbohydrate obscured the remaining portion of the spectrum. The original ellipticinium possesses an unsymmetrical carboxy hydrazide C=O at 1652 cm^{-1} and the CMCL possesses its C=O at 1750 cm^{-1} whereas the new conjugate displays C=O bands at 1728, 1694 and 1652 and none at 1750 cm^{-1} .
20 ^1H NMR (DO) 1.52-1.54, 1.79, 1.96, 3.47-4.60(Br), 4.11, 6.66, 7.24, 7.64, 7.79, 7.95, 8.95 ppm. FT-IR (KBr disk): 1694, 1651 cm^{-1} . UV (DO): 430 (Br weak), 365 (Br weak), 305 (strong), 245 (weak) nm. mp > 300 °C. d.s. = 0.13 by the method above; by variation of reaction ratios a range of (ds) from 0.01 to 0.4 can be achieved.

EXAMPLE 13

Preparation of CMC-mitoxantrone conjugate

80 mg of mitoxantrone hydrochloride salt (blue powder) were dissolved in 30 ml of anhydrous acetonitrile upon addition of 2 equivalents of triethylamine. 145 mg of CMCL were added and the reaction was carried out at reflux for 22 hr under argon. The acetonitrile was evaporated off and the excess mitoxantrone was

5 dialyzed against a 0.001N noah solution for 5 days with 2 changes of solution. This is done so as to ensure that the non-attached mitoxantrone transverses the dialysis bag because it is not very soluble in water. The retentive obtained after dialysis against the noah solution was further dialyzed for 3 days in distilled water with 2 changes of water to remove excess noah. The CMC-mitoxantrone conjugate (blue
10 flakes) was dried via lyophilization. Quantification was carried out against a calibration curve prepared with authentic mitoxantrone.

1H NOR (DO); 1.09 (m), 2.85 - 3.93 (Br), 6.75 (d). FT-IR (KBr disk); 1720, 1645, 1600, and 1590 cm^{-1} , UV (water); several weak bands, maximum at 604 nm, mp > 300 °C., d.s = 0.1 (range obtainable by adjusting reaction ratios 0.01 to 0.25

15 EXAMPLE 14

Preparation of the CMC-mitoguazone conjugate

A 100 ml round bottom flask was charged with a suspension of 75 mg (408 mmol) of mitoguazone, 50 mg of CMC-lactone (or CMCL), and 10 ml of anhydrous acetonitrile. The reaction medium was refluxed and stirred for 24 hours, filtered, and
20 the solid washed on the filter with a minimum of cold water. The solid was briefly dried in vacuum to ensure removal of the organic solvent (residual acetonitrile can dissolve holes in the dialysis bag into which the conjugate was dissolved in 80 ml of distilled water). Dialysis was conducted for five days with three changes of the external water. Lyophilization produced an off-white conjugate of no-defined melting
25 point (decomposition over a temperature range in excess of 100 °C). Mitoguazone itself acts like a pH indicator changing from a clear, nearly colorless solution to a bright yellow solution as the pH is raised from the acid range to 11. The purified carboxymethyl cellulose conjugate of mitoguazone evidenced this same pH-related color change also at pH 11. Even though this color transition can be
30 spectroscopically quantified by weighed standards of the free drug and used to

5 determine degree of substitution, the most sensitive determination is by combustion analysis to quantify nitrogen content of the dried conjugates. The parent CMCL, of course, has no nitrogen content. The procedure described above gives a product with 7.43% nitrogen which reflects a d.s. of 0.15. When carried out with a greater excess of mitoguazone, or for a longer reflux time, or in higher boiling solvents
10 (diglyme, bp 162 °C; dioxane bp 101 °C) higher d.s. values of 0.15 to 0.30 can be obtained.

EXAMPLE 15

Preparation of the pectin lactone conjugate of leaf alcohol

a. From pre-prepared pectin lactone

15 A charge of 540 mg of pectin lactone and 20 ml of leaf alcohol (cis 3-hexen-1-ol) was placed in a 50 ml round bottom flask fitted with a magnetic stirrer. Although insoluble at ambient temperature most of the pectin lactone dissolved, i.e., reacted, when heated to reflux. Reflux and stirring was continued for 24 hr whereafter the excess leaf alcohol was removed by heating *in vacuo*. The resulting gum was
20 dissolved in a minimum quantity of cold methanol and filtered to remove insoluble material. Upon evaporation in *vacuo* the polymer was examined by infrared spectroscopy revealing a ca 50% esterification. No lactone remained.

b. By *in situ* lactonization and esterification on pectin

25 Into a 25 ml flask fitted with a magnetic stirrer and condenser was placed 8.0 ml of leaf alcohol (cis 3-hexen-1-ol). The alcohol was warmed to 50 °C and treated to the portionwise addition of 200 mg of purified, dried, powdered pectin acid. The temperature was raised to 60 °C, and 4.0 ml of anhydrous toluene were added. The mixture was then heated to distill volatiles from the medium. Four ml of additional toluene were added to replace that which distilled and the distillation continued. After
30 6 hrs of heating the mixture was cooled to room temperature and a solid

5 carbohydrate precipitated (IR showed few ester and many carboxylic acid carbonyls in the solid). The solid was washed on the filter with 10 ml of hexane and the combined organic fractions distilled (30 °C at 0.6 Torr) to remove both hexane and leaf alcohol. The light yellow oil was shown by FT IR to possess 50% ester/acid (1718 cm^{-1} ester C=O and 1670 cm^{-1} pectin acid C=O).

10 EXAMPLE 16

Preparation of the vitamin E conjugate

15 A solution was prepared from 33.0 mg, 0.045 mmoles, of the vitamin E derivative, sodium alpha-tocopherol-sulfo-N-hydroxysuccinimide [Molecular Biosciences] in 7.0 ml of water which was filtered to remove traces of insoluble material. The fluid volume was raised to 15 ml by the addition of distilled water and 10 mg of carboxymethylcellulose hydrazide, pre-dissolved in 7.0 ml of water, were added. The resulting solution was stirred and heated at 50°C for 24 hrs and then at 25°C for a second 24 hr period during which a white precipitate of the vitamin E conjugate formed. Quantitative uv spectroscopy showed that the d.s. of vitamin E on the
20 carboxymethylcellulose hydrazide was 0.1.

EXAMPLE 17

Preparation of the diethylenetriaminopentaacetic acid conjugate

25 20 mg, 0.056 mmoles, of the bis-anhydride of diethylenetriaminopentaacetic acid, 10 ml of anhydrous pyridine, and 10 mg of carboxymethylcellulose hydrazide were charged to a 25 ml round bottom flask and heated to 115 °C for 24 hrs. The cellulose hydrazide did not dissolve until 10 ml of distilled water were added. Evaporation in vacuum and dialysis of the crude solid (5 days, 3 water changes to remove the non-conjugated DTPA) gave the conjugate after lypophilization. The d.s. was 0.05.

5 For the formulation of suitable products from the conjugates according to the present invention, as described above, and for all the mentioned carboxy- and carboxymethyl- saccharide conjugates, nonreacted lactone is hydrolyzed with base and dialyzed to neutrality, after which the conjugate is retained as an aqueous solution, lyophilized powder, gum, or film. Since all conjugates retained uncoupled
10 carboxylic acid moieties, they may be adjusted to their sodo salts by pH adjustment following well known protocols. This variability gives flexibility in the form for use of the conjugates according to the present invention. Forms for use comprise, but are not limited to, injectable aqueous solutions as pro-drugs for therapy, mechanically implanted strips as pro-pharmaceutical depots, solid or solution additives as pro-
15 flavors to food, solid or solution additives as pro-perfumes to laundry products, ribbons or films employed in fulfillment of the clinical objectives of adhesion inhibition or malignant growth suppression in interperitoneal cavities. Of course, by referring to these conjugates as "pro-" compounds, it is meant that the active moiety, be it pharmaceutical, flavorant, fragrance or other end-use product, is in a generally
20 inactive form while part of the conjugated material, and becomes 'active' when released from the conjugate. Conjugates of vitamin E may be used to promote local or system wound healing. Conjugates of DTPA may prove useful as either their gadolinium chelates for magnetic resonance imaging (MRI) or as their radiometal chelates for nuclear medicine imaging.

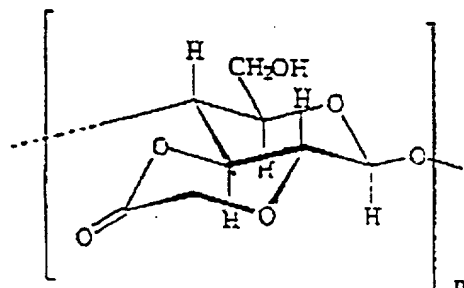
25 Thus, while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification, and we therefore do not wish or intend to be limited to the precise terms set forth, but desire and intend to avail ourselves of such changes and modifications which may be made for adapting the present invention to various
30 usage's and conditions. Accordingly, such changes and modifications are properly

10

Figure 1 consists of 11 panels (a-k) showing Western blot analysis of protein expression in C2C12 myoblasts. Each panel includes a molecular weight marker on the left and a graph on the right showing the relative protein level (fold increase) for three conditions: Control (white bar), 100 ng/ml TGF-β1 (black bar), and 100 ng/ml TGF-β1 + 100 ng/ml SB415286 (grey bar). The proteins are: (a) p38, (b) p38α, (c) p38β, (d) p38γ, (e) p38δ, (f) p38ε, (g) p38ζ, (h) p38η, (i) p38θ, (j) p38ι, and (k) p38κ. The graphs show that TGF-β1 treatment increases the expression of all p38 isoforms, and this increase is significantly inhibited by the inhibitor SB415286.

5 WE CLAIM:

1. A carboxymethyl cellulose compound according to the formula:



wherein "n" is an integer from 500 to 2000.

2. A carboxymethyl cellulose compound according to Claim 1 wherein "n" is an integer from 1000 to 15000.

3. A method for the synthesis of a lactone of polysaccharide carboxylic acids which comprises (i) providing the free acid form of the polysaccharide as a finely-powdered, anhydrous carboxylic acid with minimal sodium and potassium carboxylate content; (ii) lactonizing said polysaccharide by thermal dehydration in an anhydrous non-nucleophilic solvent; and (iii) collecting the resulting lactone product.

4. A method according to Claim 3 in which the polysaccharide carboxylic acid is selected from the group consisting of carboxymethyl cellulose, carboxymethyl alpha- and beta-dextran, carboxymethyl starch, chitosan, O,N-Carboxymethyl, O-carboxymethyl, N-carboxymethylchitosan, carboxy-starch, and pectin.

5. A method according to Claim 3 which further comprises conducting said lactonization in a refluxing media selected from the group consisting of xylene, toluene, diglyme, and acetonitrile.

6. A method according to Claim 5 wherein the polysaccharide carboxylic acid is carboxymethyl cellulose and lactonizing consists of (i) suspending the carboxymethylcellulose in anhydrous diglyme, (ii) heating the suspension to about 150

5 °C for about 24 hours, (iii) removing the diglyme solvent, and (iv) collecting the lactone.

7. A method according to Claim 5 wherein the polysaccharide carboxylic acid is pectin acid lactonizing consists of (i) suspending the pectin in anhydrous toluene, (ii) heating the suspension for about 24 hours, (iii) removing the toluene solvent, and (iv)
10 collecting the lactone.

8. A method according to Claim 5 wherein the polysaccharide carboxylic acid is carboxymethyl starch and lactonizing consists of (i) converting the starch to the free acid, (ii) suspending the free acid in anhydrous diglyme, (iii) heating the suspension, (iv) removing the diglyme solvent, and (v) collecting the lactone.

15 9. A polysaccharide carboxylic acid lactone product made in accordance to the method of Claim 3.

10. A method of forming a polysaccharide carboxylic acid lactone conjugate which comprises (i) ring-opening a polysaccharide carboxylic acid lactone selected from the group consisting of carboxymethyl cellulose, carboxymethyl alpha- and beta-dextran, carboxymethyl starch, chitosan, O,N-Carboxymethyl, O-carboxymethyl, N-carboxymethylchitosan, carboxy-starch, and pectin lactones; and (ii) coupling said
20 lactone with a compound having a biological response within a targeted end-user.

11. A method according to Claim 10 wherein ring opening is accomplished with a nucleophilic compound.

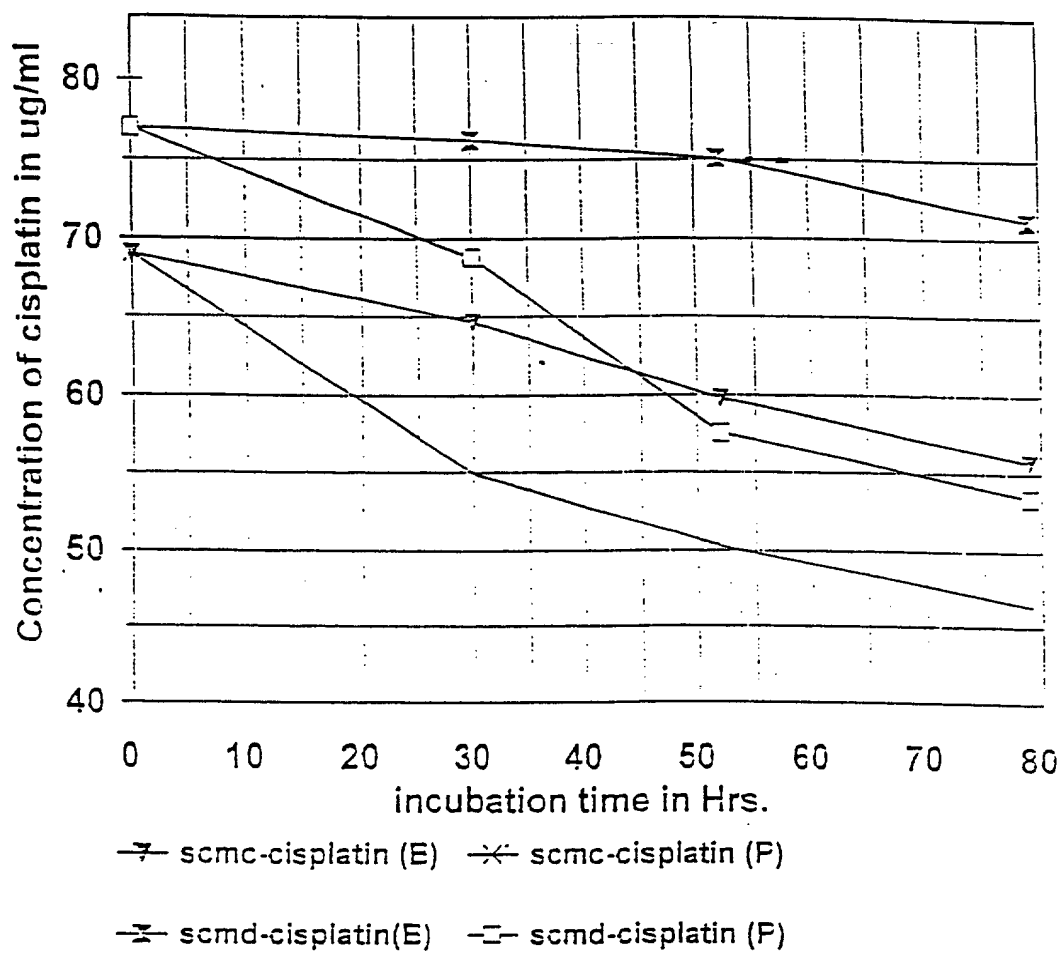
25 12. A method for the synthesis of a therapeutic compound comprising the step of conjugating a therapeutic agent to a carboxymethyl polysaccharide utilizing a reactive lactone of said carboxymethyl polysaccharide.

13. A method according of Claim 12 wherein the carboxymethyl polysaccharide is carboxymethyl cellulose, and wherein the therapeutic agent is selected from the
30 group consisting of cisplatin, ellipticinium, aminoglutethemide, mitoxantrone, finasteride,

5 vitamin E, alpha-difluoromethylornithine, mitoguazone, and nucleophilic
chemotherapeutic agents.

14. A method for the synthesis of a conjugated polymer comprising the step of conjugating an agent to a carboxymethyl polysaccharide utilizing a reactive lactone of said carboxymethyl polysaccharide, and wherein said agent is selected from the group consisting of an imaging diagnostic capable of binding radioactive metal ions for nuclear imaging or paramagnetic metal ions for magnetic resonance imaging, fragrances, flavorants, cis 3-hexen-1-ol, and property modifiers.

[illegible]



(P) - pH 7.4 Phosphate Buffer
 (B) - Fresh Rat Serum

FIGURE 1